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Request for grant of a patent

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1. Your reference — 87420/JND — 18 MAY 1998

2. Patent application number
(The Patent Office will fill in this part) — **9810681.8**

3. Full name, address and postcode of the or of each applicant (underline all surnames)

Royal Free Hospital School of Medicine
13 Rowland Hill Street
London NW3 2PF

Patents ADP number (if you know it)

If the applicant is a corporate body, give the country/state of its incorporation

4. Title of the invention

HORMONE

5. Name of your agent *(if you have one)*

PAGE WHITE & FARRER

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~~Page White & Farrer~~
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Country	Priority application number *(if you know it)*	Date of filing *(day / month / year)*

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Number of earlier application	Date of filing *(day / month / year)*

8. Is a statement of inventorship and of right to grant of a patent required in support of this request? *(Answer 'Yes' if:*
a) any applicant named in part 3 is not an inventor, or
b) there is an inventor who is not named as an applicant, or
c) any named applicant is a corporate body
See note (d))

Yes

Patents Form 1/77

9. Enter the number of sheets for any of the following items you are filing with this form. Do not count copies of the same document

Continuation sheets of this form	
Description	12
Claim(s)	2
Abstract	1
Drawing(s)	7+7

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Priority documents	Not required
Translations of priority documents	Not required
Statement of inventorship and right to grant of a patent (Patents Form 7/77)	No
Request for preliminary examination and search (Patents Form 9/77)	No
Request for substantive examination (Patents Form 10/77)	No
Any other documents *(please specify)*	No

11. I/We request the grant of a patent on the basis of this application.

Signature Page White Date 18 May 1998

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12. Name and daytime telephone number of person to contact in the United Kingdom — Mr J N Daniels 0171 831 7929

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HORMONE

The present invention relates to a hormone which is involved in the regulation of phosphate metabolism, to fragments of that hormone and to medicaments relating thereto.

Phosphate plays a central role in many of the basic processes essential to the cell and the mineralisation of bone. In particular, skeletal mineralisation is dependent on the regulation of phosphate and calcium in the body and any disturbances in phosphate-calcium homeostasis can have severe repercussions on the integrity of bone. In the kidney, phosphate is lost passively into the glomerular filtrate and is actively reabsorbed via a sodium (Na^+) dependent phosphate cotransporter. The liver, skin and kidney are involved in the conversion of vitamin D3 to its active metabolite, calcitriol, which plays an active role in the maintenance of phosphate balance and bone mineralisation.

Vitamin D deficiency causes rickets in children and osteomalacia in adults. Both conditions are characterised by failure of calcification of osteoid, which is the matrix of bone. There are also several non-dietry conditions which can lead to rickets, including X-linked vitamin D resistant hypophosphataemic rickets (HYP), hereditary hypercalciuria with hypophosphataemic rickets (HHRH), Dent's disease including certain types of renal Fanconi syndrome, renal 1 alpha-hydroxylase deficiency (VDDR I), defects in 1,25-dihydroxy vitamin D3 receptor (end organ resistance, VDDR II), and oncogenic hypophosphataemic osteomalacia (OHO).

Rowe *et al* (1996) have reported candidate 56 and 58 kDa protein(s) responsible for mediating renal defects in OHO [Rowe *et al*, Bone, 18, 159 to 169 (1996)]. A patient with OHO was treated by tumour removal and pre- and post-operative antisera from the patient were used in a Western blotting

identification of tumour conditioned media proteins. Neither the tumour cells nor the antisera were ever made available to the public, however.

In a review in Exp Nephrol, 1997, 5; 335-363, Rowe (1997) discusses the above diseases and the role of the PHEX gene (previously known as the PEX gene). The PHEX gene product has been identified as a zinc metalloproteinase and Rowe has postulated the existence of a new hormone, phosphatonin, the role of which is to regulate phosphate metabolism. As set out in attached Figure 6, Rowe has postulated that in healthy individuals phosphatonin is cleaved by the PHEX metalloproteinase and a cleaved fragment of phosphatonin binds a renal receptor. In disease states such as familial rickets, defective PHEX results in uncleaved phosphatonin which would result in down regulation of the sodium dependent phosphate cotransporter and up regulation of renal mitocondrial 24-hydroxylase. It has been speculated that a possible candidate for the phosphatonin hormone is stanniocalcin, which is known from fish. Rowe (1997) indicates that the presence of a stanniocalcin-like hormone in human kidney has been confirmed by the use of fish stanniocalcin antibodies [Wagner *et al*, PNAS (1995) 92, 1871 to 1875]. No purification of phosphatonin or stanniocalcin was reported by Rowe (1997).

In one aspect, the present invention provides phosphatonin in substantially pure form. Prior to the present invention no source material for phosphatonin was made available to the public. Moreover, purification, identification and characterisation of phosphatonin has not been possible.

The phosphatonin typically has an approximate molecular weight of 53 to 60 kDa, more preferably 58-60 kDa, as measured on SDS-PAGE, particularly on a 12.5% gel at pH7.2 in phosphate buffer (10mM). An approximate molecular weight of 200 kDa may

be measured on bis- tris-SDS-PAGE at pH7 using a 4-12% gradient gel with MOPS running buffer. It is possible on such a gel also to see lower molecular weight bands of 53 to 60 kDa. The phosphatonin is generally glycosylated.

Surprisingly, it has been found that the phosphatonin is obtainable, following purification from Saos-2 cells, which are available from the European Collection of Cell Culture under Deposit No. ECACC 89050205. Accordingly, in a further aspect of the invention, there is provided use of Saos-2 cells or HTB-96 cells for the production of phosphatonin. Other transformed or immortalised cell lines may be capable of overexpression of phosphatonin, such as transformed osteoblast or bone cell lines.

In a further aspect, the present invention provides a fragment of phosphatonin which is capable of regulating phosphate metabolism, preferably by up-regulating renal sodium dependent phosphate cotransport and/or down-regulating 25-hydroxy vitamin D3-24-hydroxylase. These activities are readily measurable using the methodology of Rowe *et al* (1996) by assay using a suitable renal cell line such as CL8 or OK (deposited at the European Collection of Cell Cultures under ECACC 91021202). Preferably, the fragment is obtainable by proteolytic cleavage of phosphatonin by a PHEX metallopeptidase. A PHEX gene has been cloned and found to encode a zinc metallopeptidase as discussed in Rowe (1997).

In a further aspect, the present invention provides phosphatonin or a fragment thereof, for use as a medicament. In particular, fragments of phosphatonin as described above may be useful as a medicament in the treatment of a disorder of phosphate metabolism such as X-linked rickets and osteomalacia as well as other diseases of bone mineral metabolism. There is further provided phosphatonin and PHEX metallopeptidase as a combined preparation for simultaneous,

separate or sequential use as a medicament. In this way, the PHEX metallopeptidase may be used to cleave phosphatonin so as to produce active phosphatonin fragments which may be used for the treatment of disorders of phosphate metabolism as discussed herein.

The present invention has therefore provided for the first time phosphatonin in a substantially isolated or purified form which is suitably free of contaminants. Native phosphatonin and native fragments of phosphatonin, which are free of contaminants such as SDS and/or other interfering proteins are capable of regulating phosphate metabolism and of providing active ingredients in pharmaceutical compositions for the treatment of diseases associated with disorders of phosphate metabolism.

The present invention will now be described in further detail, by way of example only, with reference to the accompanying drawings, in which:

FIGURE 1 shows a photomicrograph of an immortalised tumour cell line capable of expressing phosphatonin;
FIGURE 2(a) and (b) show respectively chromatograms with low affinity and high affinity protein-containing peaks from a concanavilin A column;
FIGURE 3 shows a cation exchange chromatogram of fractions from the concanavilin A column;
FIGURE 4 shows results of Western blotting of material from the concanavilin A column;
FIGURE 5 shows results of Western blotting and glycoprotein detection of tumour condition medium and purified fractions using enhanced chemiluminescence;
FIGURE 6 shows the results of Western blotting on an SDS-PAGE polyacrylamide gradient gel; and
FIGURE 7 shows a simplified scheme for the roles of phosphatonin and PHEX in phosphate metabolism.

Examples

A mesenchymal tumour with phosphaturic expression was removed from a patient and the following samples taken:

A: Sample of pure tumour tissue, size of two large peas, was placed into a 2 ml vial containing DMEM Eagles/ 10%FCS/Glutamine/antibiotic antimycotic Gibco-BRL.

B: Sample of sub-dura tumour approximately the same size possibly smaller. Placed in same media as A.

C: Sample of abnormal dura: tough white material: Placed in same media as A.

D: Sample of tumour fluid.

Processing of Samples:

DAY 1

The samples were each cut into small 0.5 cm cubes using a sterile scalpel. Half of each sample was placed into a cryotube and frozen down in N2(l) immediately. The fluid surrounding the tissue (DMEM/10% FCS etc.), was also collected and frozen down. The other half of each sample was added to DMEM Eagles/10% FCS/Glutamine/Antimycotic antibiotic supplemented with collagenase A1 0.2mg/ml (~15ml), and left at 37 C O/N.

DAY 2

1. After overnight incubation in serum supplemented DMEM, the cells appeared to be predominantly RBC's and very few adherent cells were observed. The cells were spun down at room temp and the supernatants collected and immediately frozen down (~15ml).

2.The pellets were then resuspended in 10 ml of DMEM Eagles supplemented with antibiotic/antimycotic (medium flasks), and then incubated for a further 8h 10 min.

3.The serum-free supernatants were collected as described in 1 (~10 ml), and the cells were resuspended in DMEM EAGs with 10% FCS etc., (~15 ml), and incubation continued. The supernatants were stored at -80°C.

DAY 6

1.After incubation from Day 2, cells were spun down as described for 1 of Day 2. 10% FCS samples were collected and frozen.

2.Pellets were resuspended in serum free DMEM (10 ml), as for Day 2. and this time left for four hours.

3.Same as for 3 of Day 2.

DAY 7:

1.The subdura and tumour culture in particular, had developed innumerable foci containing clumps of cells which appeared attached to the plastic of the tissue culture plates. Underneath these polyp like protuberances was a monolayer of fibroblast like cells which spread out radially from underneath the tumour like structures. This layer of cells appeared to act as a matrix to anchor the polyp like tumours. None of this was seen in the dura sample, which appeared to lack cells at this stage, and contained fibrous like matted structures.

2.Cultures were spun down, and the supernatants collected (10% FCS). The pellets were then placed to one side.

3. The plates were then incubated with 10 ml of trypsin EDTA soln Gibco/BRL 1/10 dilution in PBS for ~15 min. Plates were then tapped vigorously and 5 ml of FCS added.

4. The resuspended cells were then added to the pellets obtained in 2, resuspended and spun down. The supernatant was discarded.

5. Cells were then plated out in 18 ml of 10 % FCS DMEM Eagles medium with glutamine and antibiotic antimycotic supplements (large flasks were used).

6. Finally cells were incubated at 37 C in a CO2 atmosphere.

DAY 9:

1. Tumour cells and to some extent the subdura cells appeared as innumerable clumps of cells, and appeared to have the same morphology as the cells prior to trypsin treatment. Some of the clumps were quite large, and visible to the naked eye.

2. The serum supplemented media was collected and stored down. Large flasks were used and 18 ml of media per flask added (DMEM 10% FCS antimycoctic/antibiotic/glutamine).

DAY 13:

1. Cells were frozen down (~15 ml), and stored in falcons as 10%FCS DMEM conditioned media.

2. Cells resuspended in serum free DMEM Eags (~11 ml) 11.10 am, and left for 6 h at 37°C (CO2 incubator).

3. Cells were then spun down and the supernatants collected (serum free control media). 10% FCS DMEM EAG was then added to the remaining cells.

DAY 16

The above process was repeated and Tumour Conditioned Medium (TCM) collected over several weeks.

Alternatively, TCM may be collected from Saos-2 cells (ECACC 89050205) or U-2 OS cells (ATCC HTB-96).

Isolation of phosphatonin:

Concanavilin A sepharose affinity chromatography:

1. 3ml of TCM was adjusted with 1M sodium phosphate pH 7.2 and 5M NaCl to give a final concentration *of* 0.06M Sodium phosphate pH 7.2 and 0.5M NaCl plus 0.01% sodium azide.
2. Con A Sepharose (Pharmacia Code No: 17-0440-01), arrived in 20% Ethanol, and this was first washed with several column volumes of water, and then equilibrated in the running buffer. A small C10/10 column (Pharmacia code No: C10/10 id 10 mm), was packed with Con A to a height of 5.5 cm (approx. volume 4.3 to 5.0 ml). Equilibration was carried out at max flow rate of 0.5 ml/min.
3. The sample (adjusted to pH 7.2 sodium phosphate/0.5M NaCl/0.01% sodium azide), was then added to the column by gravity feed, and reloaded three times. The colour of the sample enabled visualisation of the passage through the column. Unbound material was then collected and stored for future reference.
4. Waters LC system was then connected and the sample was washed with several column volumes of loading buffer.
5. After loading and washing, elution was carried out using sodium phosphate buffer 60 mM pH 7.2/ 0.5M Nacl/0.5M a-methyl-D-glucopyranoside/0.01% azide buffer.. See Figure 2a. A single peak was detected and this was collected .
6. The column was then run to base line approximately 40 ml max, and then left overnight

7. After O/n incubation in methyl glycoside buffer, a second peak was eluted (see Figure 2b), which peaked at ~5 ml.
8. The second peak was collected and dialysed against 0.05M acetic acid, and then lyophilised. Both Conacanavilin peaks A1 (low affinity), and concanavilin A2 (high affinity), are potent at inhibiting Na+ dependent phosphate co-transport and vitamin D metabolism in a human renal cell line (CL8). The high affinity fraction The human renal cell line (CL8), and the conditions used for assay are described in Rowe et al 1996. A further suitable known renal cell line for this assay is the OK cell line deposited as ECACC 91021202.

Cation exchange Chromatography using HiTrap SP cation exchange 1 ml column

(Code No 17-1151-01; Pharmacia):

1. The lyophilised protein was then re-dissolved in 0.05M ammonium acetate pH 5 and the applied to an equilibrated 1 ml HiTrap SP sepharose cation exchange column.
2. The column was equilibrated prior to sample addition by washing with water, and then 5 volumes of start buffer (0.02 M ammonium acetate pH 5).
3. Sample was eluteD using the following protocol;

Num	Time min	Flow rate ml/min	%NH_4 acetate pH 5	% NH_4 acetate/ 0.5M NaCl pH 5
1		0.5	100	0
2	15	0.5	25	75
3	20	0.5	0	100
4	25	0.5	0	100
5	35	0.5	100	0
6	50	0.5	100	0

A single sharp peak was obtained, and the sample was then dialysed against 0.05M acetic acid and lyophilised. See Figure 3.

After resuspending in 10 mM phosphate buffer pH 7.2 20 ul, 10 ul was run on an SDS PAGE gel 12.5% (see chromatogram), and a double band of 55 KD was resolved. Both the Concanavilin A and cation bands also have an aggregated form All fractions including the tumour conditioned media were potent at inhibiting Na+dependent phosphate co-transport in a human renal cell line (1/1000 diln), and also altered vitamin D metabolism. For a full description of the methods used to measure phosphate transport and vitamin D metabolism see Row *et al* 1996. All purification modalities were carried out on a waters HPLC/FPLC system programmed by computer-millennium software. The most active fraction was the concanavilin A1 peak shown in figure 4, which shows Western (chemiluminescence) of purified concanavilin A1 fraction from OHO tumour. Anti pre-operation antisera was used to screen the immobilised purified fraction. The fraction is also potent and inhibiting NaPI, and affects vitamin D metabolism in a human renal cell line (CL8).

Screening of tumour conditioned-medium (TCM), and purified fractions with pre/post— operation antisera: plus glycoprotein screen.

Pre-operation and post-operation antisera from a patient has been described previously in Rowe *et al* 1996. Only pre-operation antisera detected the purified fractions and hormone in TCM as shown in figure 5 in which Western and glycoprotein detection of TCM and purified fractions was achieved using enhanced chemiluminescence. Protein markers were biotinylated, and tagged with strepavidin peroxidase conjugate . The arrows show the aggregate and active glycoprotein. Post-operation antisera and rabbit pre-immune sera did not detect any of the fractions. Also, only those tumours secreting phosphaturic factor were positive. Media and skin controls were negative. A distinct feature of the Con A1, Con A2 and CA1 samples was

their potent ability to inhibit NaPI, and alter vitamin D metabolism in a human renal cell line (CL8). All the purified fractions have a tendency to aggregate into a lower mobility form on SDS-PAGE, as seen in figure 5. Also, the purified fractions and TCM active fractions are heavily glycosylated. The extent of glycosylation was confirmed by periodate oxidation of immobilised proteins on PVDF membranes followed by biotinylation of carbohydrate moieties. These were then screened with streptavidin conjugated to horse radish peroxidase and enhanced chemiluminescence (figure 5). The active form (inhibits NAPI etc.), is associated with the 58 to 60 kDa fraction shown in figure 5. An additional and powerful way of purifying the protein to homogeneity is the use of a neutral pH 7 SDS-PAGE system using a 4-12% Bis-Tris Gel with MOPS running buffer. Pre-caste gels can be purchased from Novex.

SDS-PAGE at neutral pH using 4-12% polyacrylamide gradient and Bis-Tris gel with MOPS running buffer (Nu-PAGE system from NOVEX): Reduced mobility of hormone.

On this system a fraction of the glycosylated hormone has a reduced mobility, and runs at ~200 kDa. The lower molecular weight form is also visible at 58/60 kDa. Appearance of the ~200kDa protein may be due to the isoelectric point of the protein (different charge at neutral pH), and the interaction of carbohydrate moiety with the gel matrix. Also, increased efficiency of electro-blotting of high molecular weight components occurs due to the low % acrylamide (4-12% gradient), at the top of the gradient gel. Running fractions through this system increases the purity and homogeneity of the molecule. Western blot (ECL) of conditioned media from tumour cell lines, and osteoblast cell lines (pre-operation antiserum from OHO patient). 1: protein markers; 2. intracranial tumour cell line OHO; 3 cells from sub-dura adjacent to tumour; 4. Cells from dura adjacent to sub-dura;

5. HTB6 cell line; 6. Saos-2 cell line; 7. defined medium control; 8. Skin fibroblast control; 9. Linear seabaceous naevus polyp tumour. The specific phosphaturic band at ~200kDa on SDS-PAGE Neutral gels is highlighted in the Figure. The above is representative; many control experiments have been done using a number of tumours (phosphaturic/non-phosphaturic).

CLAIMS:

1. Phosphatonin in substantially pure form.

2. Phosphatonin according to claim 1, which has an approximate molecular weight of 53 to 60 kDa as measured on SDS-PAGE.

3. Phosphatonin according to claim 1 or claim 2, which has an approximate molecular weight of 200kDa as measured on bis-tris SDS-PAGE at pH7.

4. Phosphatonin according to any one of claims 1 to 3, which is glycosylated.

5. Phosphatonin according to any one of the preceding claims, which is obtainable following purification from Saos-2 cells (Deposit No. ECACC 89050205).

6. A fragment of phosphatonin according to any one of the preceding claims, which is capable of regulating phosphate metabolism.

7. A fragment according to claim 6, which is capable of up-regulating renal sodium dependent phosphate co-transport.

8. A fragment according to claim 6 or claim 7, which is further capable of down-regulating 25-hydroxy vitamin D3-24-hydroxylase.

9. A fragment according to any one of claims 6 to 8, which is obtainable by proteolytic cleavage of phosphatonin by a PHEX metallopeptidase.

10. Phosphatonin or a fragment thereof, according to any one of the preceding claims, for use as a medicament.

11. Phosphatonin and PHEX metallopeptidase as a combined preparation for simultaneous, separate or sequential use as a medicament.

12. Use of phosphatonin or a fragment thereof, according to any one of the preceding claims, for the preparation of a medicament for treatment of a disorder of phosphate metabolism.

13. Use of phosphatonin and PHEX metallopeptidase for the manufacture of a combined preparation for simultaneous, separate or sequential use for the treatment of a disorder of phosphate metabolism.

14. Use of a transformed osteoblast or bone cell line capable of phosphatonin overexpression for the production of phosphatonin.

15. Use according to claim 14 wherein the cell line is an Saos-2 cell (Deposit No. ECACC 89050205) or a U-2 OS cell (Deposit No. ATCC HTB-96).

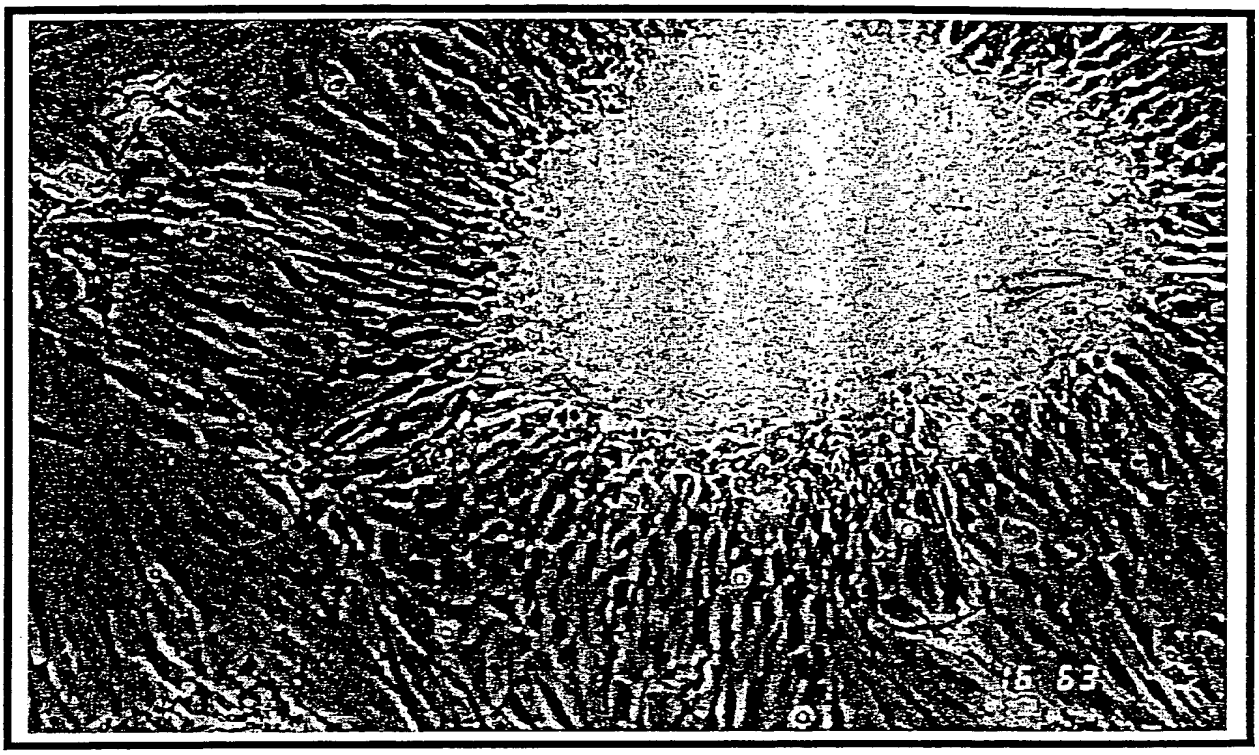


FIGURE 1

09-Au-at M

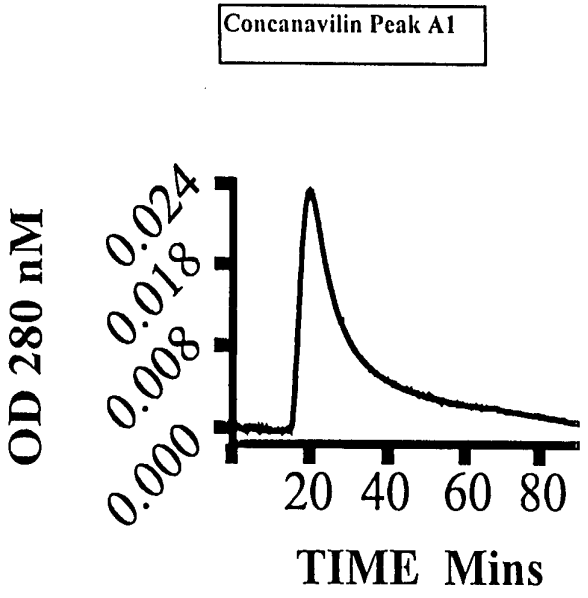


Figure 2a

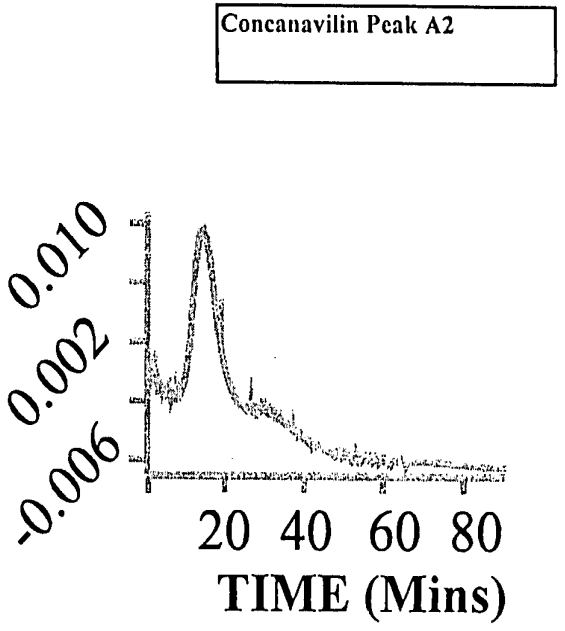


Figure 2b

09-Oct-91 M

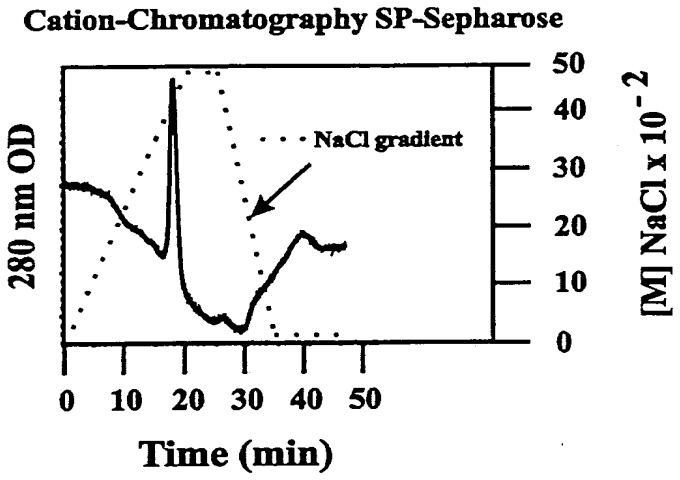


FIGURE 3

09-Ju-91 M

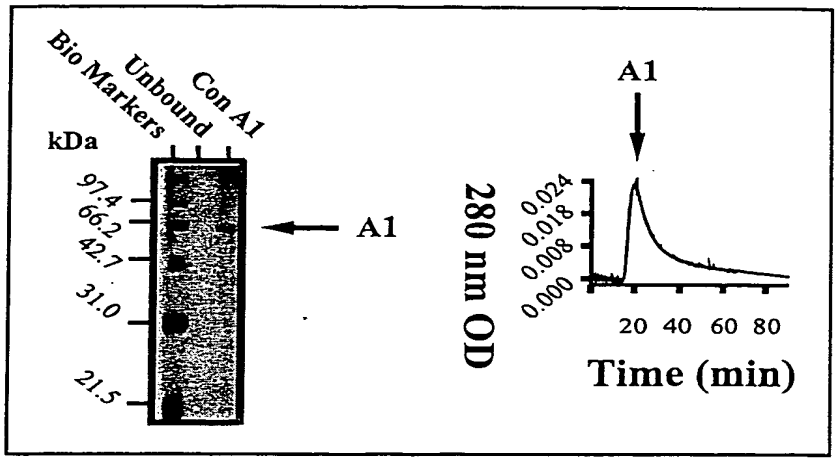
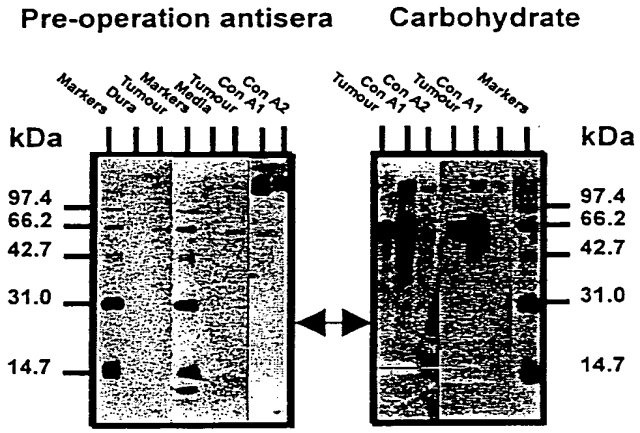

Bio Markers
Unbound
Con A1
kDa
97.4
66.2
42.7
31.0
21.5
A1
A1
280 nm OD
0.024
0.016
0.008
0.000
20 40 60 80
Time (min)

FIGURE 4

09-Au-81 M


Pre-operation antisera
Carbohydrate
Markers
Dura
Tumour
Markers
Media
Tumour
Con A1
Con A2
Tumour
Con A1
Con A2
Tumour
Con A1
Markers
kDa
97.4
66.2
42.7
31.0
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kDa
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66.2
42.7
31.0
14.7

FIGURE 5

09:00 AM

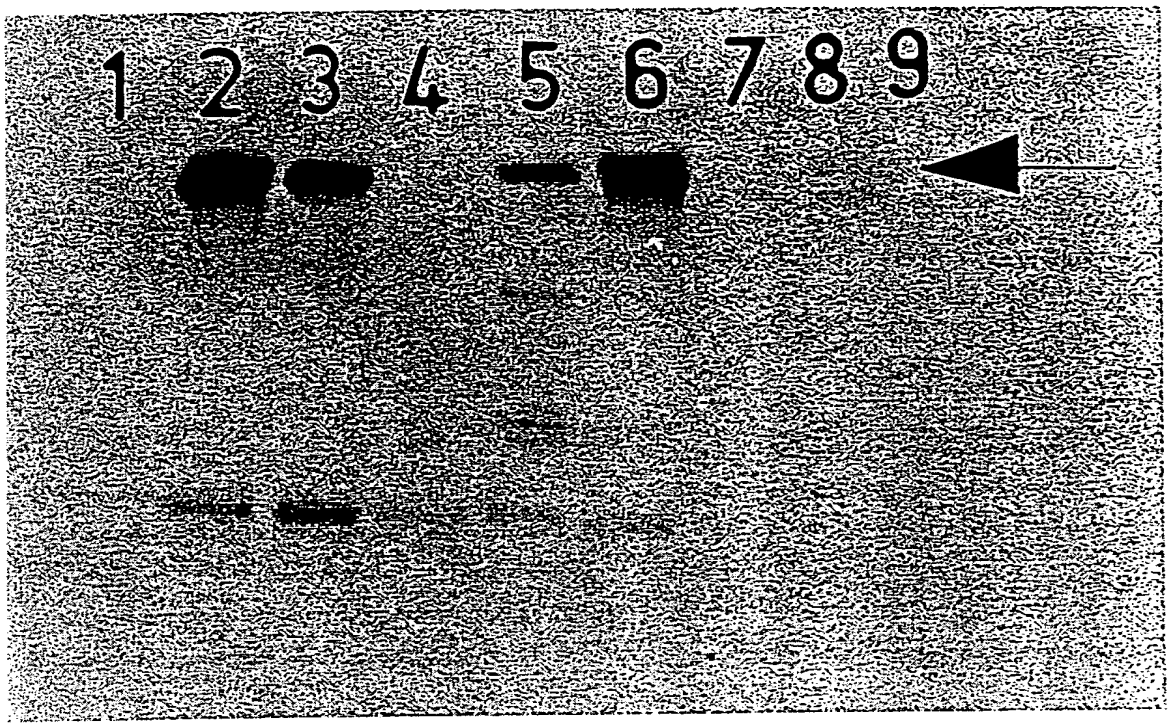
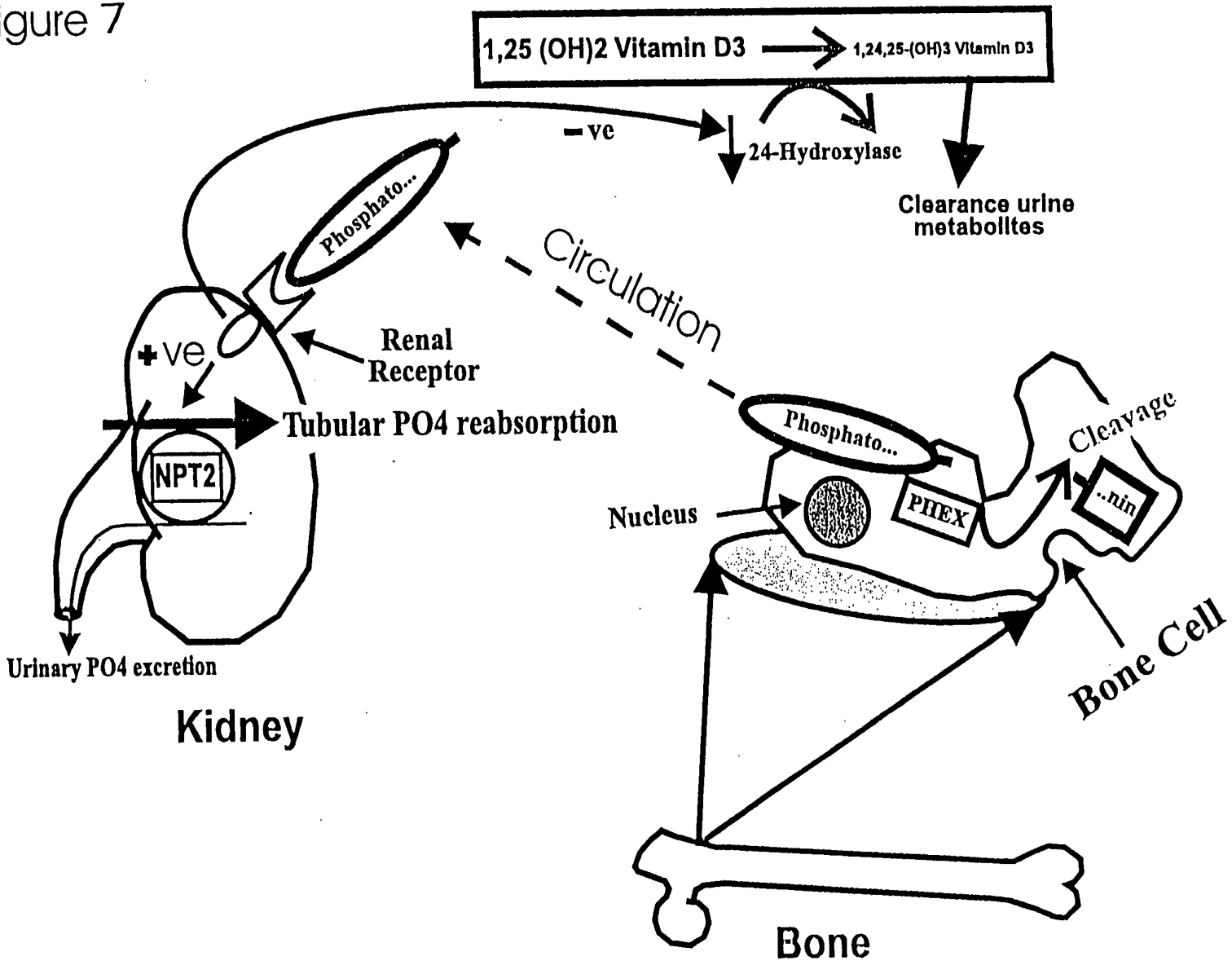


FIGURE 6

09-Au-81 M

Figure 7


1,25 (OH)2 Vitamin D3
1,24,25-(OH)3 Vitamin D3
-ve
24-Hydroxylase
Clearance urine metabolites
Phosphato...
Circulation
+ve
Renal Receptor
Tubular PO4 reabsorption
NPT2
Urinary PO4 excretion
Kidney
Phosphato...
Cleavage
Nucleus
PHEX
..nin
Bone Cell
Bone

99-09-31 M